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Human Interleukin-3 (IL-3) Induces Disulfide-Linked IL-3 Receptor α- and β-Chain Heterodimerization, Which Is Required for Receptor Activation but Not High-Affinity Binding

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The human interleukin-3 receptor (IL-3R) is a heterodimer that comprises an IL-3-specific α chain (IL-3Rα) and a common β chain (β_c) that is shared with the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. These receptors belong to the cytokine receptor superfamily, but they are structurally and functionally more related to each other and thus make up a distinct subfamily. Although activation of the normal receptor occurs only in the presence of ligand, the underlying mechanisms are not known. We show here that human IL-3 induces heterodimerization of IL-3Rα and β_c and that disulfide linkage of these chains is involved in receptor activation but not high-affinity binding. Monoclonal antibodies (MAb) to IL-3Rα and β_c were developed which immunoprecipitated, in the absence of IL-3, the respective chains from cells labelled with ^{125}I on the cell surface. However, in the presence of IL-3, each MAb immunoprecipitated both IL-3Rα and β_c. IL-3-induced receptor dimers were disulfide and nondisulfide linked and were dependent on IL-3 interacting with both IL-3Rα and β_c. In the presence of IL-3 and under nonreducing conditions, MAb to either IL-3Rα or β_c immunoprecipitated complexes with apparent molecular weights of 215,000 and 245,000 and IL-3Rα and β_c monomers. Preincubation with iodoacetamide prevented the formation of the two high-molecular-weight complexes without affecting noncovalent dimer formation or high-affinity IL-3 binding. Two-dimensional gel electrophoresis and Western blotting (immunoblotting) demonstrated the presence of both IL-3Rα and β_c in the disulfide-linked complexes. IL-3 could also be coimmunoprecipitated with anti-IL-3Rα or anti-β_c MAb, but it was not covalently attached to the receptor. Following IL-3 stimulation, only the disulfide-linked heterodimers exhibited reactivity with antiphosphotyrosine antibodies, with β_c but not IL-3Rα being the phosphorylated species. A model of IL-3R activation is proposed which may be also applicable to the related GM-CSF and IL-5 receptors.

Engagement of the human interleukin-3 receptor (IL-3R) by IL-3 triggers a variety of cellular signals resulting in the preservation of cell viability, proliferation, and differentiation of hemopoietic cells (4, 27). Expression of the IL-3R, while subject to regulation, is maintained during hemopoietic cell differentiation, and its activation on the mature cells leads to enhanced function of monocytes (10), eosinophils (26), basophils (12, 24), and neutrophils (44). The IL-3R has been shown to be expressed also on endothelial cells with activation by IL-3 stimulating cytokine release and the expression of adhesion molecules (20, 21). The wide expression of the IL-3R on hemopoietic cells and on cells of the blood system suggests roles in hemopoiesis, allergy, atherosclerosis, and chronic inflammation. However, the mechanism of IL-3R activation remains unclear.

The human IL-3R is a heterodimeric receptor consisting of an IL-3-specific α chain (IL-3Rα) (19) and a common β chain (β_c) (13) that is also a component of the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (reviewed in references 25 and 34). Both receptor chains belong to the cytokine receptor superfamily (3), although it has been noted that the IL-3Rα chain is more closely related to the GM-CSF receptor α chain (GM-CSFRα) and IL-5Rα chains than to other cytokine receptors (11). Thus, these three α chains can be recognized structurally and possibly functionally as a distinct subfamily. β_c, on the other hand, is structurally more closely related to gp130 and the IL-2Rβ chain (11) and, analogous to these common receptor subunits, it converts low-affinity ligand binding to high-affinity ligand binding and acts as a signal transducer (29).

The expression of both IL-3Rα and β_c is necessary for triggering signalling and cellular proliferation in response to IL-3 (18). Stimulation of cells with IL-3 leads to activation of JAK-2 (38, 43) and Lyn (48) kinases, phosphatidylcholine hydrolysis and protein kinase C translocation (39), activation of multiple isoforms of the signal transducer protein Stat 5 (1, 30), and gene expression (52). Although some form of receptor dimerization has been presumed to take place, the relative contribution of each receptor chain, the nature of their association, and the implications for receptor activation are not known.

Receptor dimerization is recognized to be important for activation in many receptor systems. For example, receptor tyrosine kinases (14, 22, 49), as well as nontyrosine kinase receptors such as the G-CSFR (16), undergo homodimerization following ligand binding, which leads to signalling. Homodimerization has also been observed in the erythropoietin (EPO) receptor (28) and in a mutant EPO receptor that is constitutively active (53). IL-6R and ciliary neurotrophic factor receptor (CNTFR) dimerization have also been shown to occur. These receptors are more analogous to the IL-3R in that

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they consist of a binding subunit and signalling subunits (7, 15). In these cases, ligand triggers subunit association but only dimerization of the signalling subunits; the signalling subunits, gp130 in the case of IL-6, and gp130 and the leukemia inhibitory factor-binding protein (LIFR) in the case of CNTF, mediate signalling (7, 32). In the IL-3R, GM-CSFR, and IL-5R system, however, the biochemical evidence of receptor dimerization has been missing. Furthermore, the requirements for the α chain and β_c in receptor activation based on cell lines transfected with genetically manipulated receptors is controversial. On the one hand, chimeric receptors consisting of extracellular α chains and intracellular β_c allow function in the presence of intact β_c and ligand, suggesting that β_c dimerization is sufficient for signalling (9, 33, 47). On the other hand, deletion of the cytoplasmic domain of the α chains abolishes ligand-mediated stimulation, suggesting that an intact α chain may be required for receptor dimerization and signalling (40, 54). Using primary human cells, we show here that IL-3Rα and β_c undergo dimerization following stimulation with IL-3, and that, unlike the IL-6R and CNTFR, the α chain of the IL-3R is part of the disulfide- and the non-disulfide-linked dimers. Furthermore, the disulfide-linked IL-3Rα–β_c heterodimer is shown to be required for receptor activation and phosphorylation of β_c but not for affinity conversion. Given the conservation of IL-3Rα with the GM-CSFR and IL-5Rα chains and the common nature of the β chain, the results presented here may also be applicable to the GM-CSFR and IL-5R.

MATERIALS AND METHODS

Cytokines and cells. Human IL-3, GM-CSF, and the IL-3 mutant E22R were produced in *Escherichia coli* and purified to homogeneity by reverse-phase high-pressure liquid chromatography (2). Quantitation was performed by integration of peak absorbance of protein stained with Coomassie blue R-250 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IL-3 was radiolabelled with ^{125}I by the ICl method as previously described (5). The cells used, unless otherwise indicated, were primary human leukemic cells obtained from the blood of a patient with chronic myeloid leukemia (CML cells) after separation on Ficoll-Paque (Pharmacia). CML cells were T- and B-cell antigen negative, CD33 and CD34 positive, and IL-3Rα, GM-CSFRα, and β_c positive, as judged by flow cytometry with specific monoclonal antibodies (MAb) and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig). The other two types of cells used were the human UT7 cell line and COS cells transfected with IL-3Rα and β_c cDNAs. The β_c DNA construct was cloned in pSG5 (gift from S. Barry and R. D'Andrea).

Anti-IL-3R MAb. MAb against the IL-3Rα and β chains were raised by immunizing mice with COS cells transiently transfected with the IL-3Rα or β_c cDNA and selecting on CHO cell transfectants stably expressing each receptor chain. MAb 9F5 against the IL-3Rα chain and MAb 4F3 against β_c were selected for their ability to provide a strong signal in immunoprecipitation analysis, and MAb 1C1 against β_c and MAb 9F5 were selected for giving strong signals on Western blot (immunoblot) analysis. MAb 7G3 is directed against the IL-3Rα chain and blocks IL-3 binding and IL-3-mediated functions (46). Each MAb was produced as ascitic fluid and purified by protein G Sepharose. For immunoprecipitation experiments, MAb 9F5 and 4F3 were directly coupled to Sepharose beads by using CNBr-activated Sepharose 4B as previously described (45).

Antiphosphotyrosine MAb. The MAb against phosphorylated tyrosine was the peroxidase-conjugated antiphosphotyrosine 3-365-10 (Boehringer Mannheim, Frankfurt, Germany).

Fluorescence-activated cell sorting staining. Fifty microliters of cells (10^5 cells) was added to 50 μl of optimally diluted antibody and incubated at 4°C for 30 min. The cells were washed with phosphate-buffered saline (PBS) and then incubated with 1/50 fluoresceinated rabbit anti-mouse antibody (DDAF; Silenus, Melbourne, Australia) for 30 min at 4°C. The cells were then washed and resuspended in FACS FIX (PBS with 2% glucose, 1% formaldehyde, and 0.02% sodium azide) and analyzed with a Coulter Profile Flow Cytometer (Coulter Electronics, Hialeah, Fla.).

Immunoprecipitation of the IL-3R. CML cells were surface labelled with ^{125}I. Usually 10^8 cells in 1 ml of PBS were mixed with 1 mCi of ^{125}I (NEN) and radiolabelled by the lactoperoxidase method as described previously (50). Following labelling, the cells were incubated with medium, IL-3, GM-CSF, or IL-3 mutant E22R, for different times before being lysed with lysis buffer composed of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10% glycerol, 1% Nonidet P-40, and protease and phosphatase inhibitors (10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride, and 2 mM sodium vanadate). After 30 min at 4°C, the lysate was centrifuged at 10,000 × *g* for 15 min, and the supernatant was removed, precleared with mouse Ig-coupled Sepharose beads for 18 h at 4°C, and incubated with anti-IL-3Rα MAb, anti-β_c MAb, or anti-phosphotyrosine-coupled Sepharose beads for 2 h at 4°C. The beads were then washed 6 times with lysis buffer before being boiled for 5 min in SDS-PAGE sample buffer and the immunoprecipitated proteins were separated by SDS-PAGE. In some experiments, the alkylating agent iodoacetamide was added to the cells for 20 min at 4°C before or after the cells were incubated with IL-3.

SDS-PAGE. Immunoprecipitated proteins were analyzed by one-dimensional (1D) or two-dimensional (2D) SDS-PAGE under reducing and nonreducing conditions. Separation of proteins by 1D SDS-PAGE and reducing conditions utilized 7.5% polyacrylamide linear gels and boiling in SDS sample buffer containing 4% 2-mercaptoethanol. Proteins were separated under nonreducing conditions on SDS–6% polyacrylamide linear gels and the samples were boiled in SDS buffer without 2-mercaptoethanol. Proteins separated by 2D SDS-PAGE were subjected to separation under nonreducing conditions in tube gels followed by separation under reducing conditions in the second dimension (36). For 1D gels, molecular weights (MW) were estimated with commercially available MW markers (Bio-Rad Broad Range Standards 161-0318). For 2D gels, the MW of the separated proteins were estimated by mixing the ^{125}I-labelled immunoprecipitated proteins with solubilized platelet proteins and by using the known MW of the major characterized platelet proteins visualized by Coomassie blue staining. The ^{125}I-labelled immunoprecipitated proteins were detected and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Western blotting (immunoblotting). Immunoprecipitated proteins separated by SDS-PAGE were transferred onto nitrocellulose filters by electroblotting. The filters were blocked with a solution consisting of 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.85% Tween 20 containing 5% bovine serum albumin and then probed with either anti-IL-3Rα MAb (9F5), anti-IL-3R β_c (1C1), or anti-phosphotyrosine (3-365-10) MAb followed where appropriate by goat anti-mouse Ig coupled to horseradish peroxidase. In order to detect radiolabelled immunoprecipitated proteins and Western blot reactivity on the same filters, the chemiluminescence method with an ECL kit (Amersham, Little Chalfont, United Kingdom) was used per the manufacturer's guidelines.

Scatchard analysis. Binding assays were performed with 0.5×10^5 to 1×10^5 cells in 150 μl of binding medium. Cells were incubated in the presence of a concentration range of 10 pM to 10 nM ^{125}I-labelled IL-3. Nonspecific binding was determined from samples containing 1 μM unlabelled IL-3. The nonspecific binding component for datum points obtained at lower radioligand concentrations was obtained by interpolation. After incubation at 21°C for 2 h with shaking, cell-associated radioligand was separated from free radioligand by overlaying the cell suspension on a 0.2-ml cushion of fetal calf serum and centrifuging for 10 s at maximum speed in a microcentrifuge. The visible cell pellet was removed by cutting, and radioactivity was determined with a Cobra 5010 γ-counter (Packard, Meriden, Conn.). Dissociation constants were calculated by using the EBDA and LIGAND programs (31) (Biosoft, Cambridge, United Kingdom).

RESULTS

IL-3 induces IL-3Rα and β_c association. In order to study the molecular events leading to IL-3R activation in primary cells, we developed MAb specific for IL-3Rα and for β_c which could give strong signals in immunoprecipitation and Western blot analyses. From several MAb raised against COS cell transfectants overexpressing IL-3Rα or β_c, three MAb were selected: MAb 9F5 specifically immunoprecipitated IL-3Rα from stable CHO cell transfectants, while MAb 4F3 immunoprecipitated β_c from CHO cells transfected with β_c cDNA. These MAb did not react with CHO cells expressing GM-CSFRα (data not shown). For Western blot analyses, MAb 9F5 gave a strong signal on CHO cells expressing IL-3Rα and MAb 1C1 gave the strongest signal on CHO cells expressing β_c. These MAb were used to screen several primary myeloid cells; of these cell lines, CML cells obtained from a patient with chronic myeloid leukemia exhibited the largest number of receptor molecules, as judged by flow cytometry, and were therefore used for subsequent experiments (data not shown).

In order to determine whether the IL-3R exists as a preformed complex or is induced by ligand, MAb 9F5 (anti-IL-3Rα) and MAb 4F3 (anti-β_c) were used in immunoprecipitation experiments and the proteins were separated on SDS-polyacrylamide gels under reducing conditions. We found that in the absence of IL-3, MAb 9F5 immunoprecipitated only IL-3Rα and MAb 4F3 immunoprecipitated only β_c from ^{125}I-

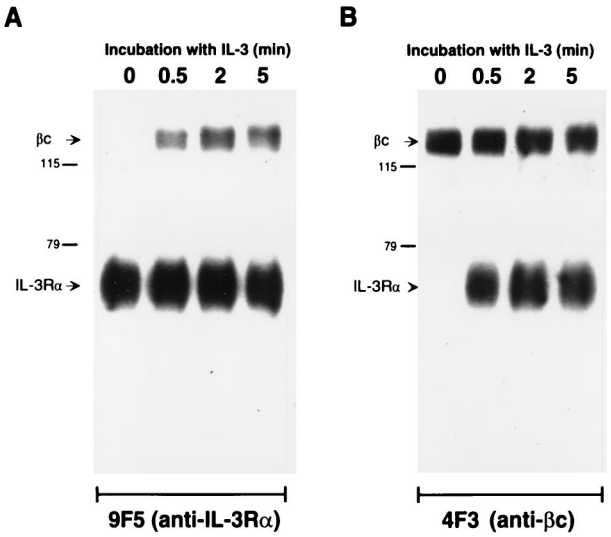

FIG. 1. IL-3 induces IL-3R complex formation. Cells labelled with ^{125}I on the cell surface were incubated with 50 nM IL-3 for different times at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti-IL-3Rα) (A) or MAb 4F3 (anti-β_c) (B), separated on SDS–7.5% polyacrylamide gels under reducing conditions, and visualized by PhosphorImaging. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

surface-labelled CML cells (Fig. 1). However, the addition of IL-3 caused the coimmunoprecipitation of a 120,000-MW protein in the case of immunoprecipitation with MAb 9F5 (Fig. 1A) and of a 70,000-MW protein in the case of immunoprecipitation with MAb 4F3 (Fig. 1B). The association of these proteins was rapid and detectable within 30 s. To determine the generality of this phenomenon, we performed further experiments using the human UT7 cell line which expresses IL-3R and COS cells transfected with the IL-3Rα and β chains. In both cases, IL-3 induced the formation of the heterodimeric complex (Fig. 2A and B).

The MW of the coimmunoprecipitated proteins suggested that they corresponded to β_c when MAb 9F5 was used and to IL-3Rα when MAb 4F3 was used (Fig. 1). To test whether this was indeed the case, we performed Western blot analyses using chemiluminescence as the detection method. In addition, and as a specificity control, the cells were incubated with GM-CSF (Fig. 3). We found that MAb 9F5 immunoprecipitated IL-3Rα together with a 120,000-MW protein in the presence of IL-3 but not in the presence of GM-CSF (Fig. 3A). Conversely, MAb 4F3 immunoprecipitated β_c and a 70,000-MW protein only in the presence of IL-3 (Fig. 3B). We consistently found a weak band with an MW of approximately 80,000 coimmunoprecipitating with β_c, and other experiments showed that this band represents GM-CSFRα (data not shown). To determine the identities of the coimmunoprecipitated bands, we probed the proteins immunoprecipitated with MAb 9F5 (anti-IL-3Rα) by Western blotting with MAb 1C1 (anti-β_c), and reciprocally, the proteins immunoprecipitated with MAb 4F3 (anti-β_c) with MAb 9F5 (anti-IL-3Rα). These data established that β_c coimmunoprecipitated with IL-3Rα and IL-3Rα coimmunoprecipitated with β_c, but only in the presence of IL-3 (Fig. 3B).

IL-3R complex formation is dependent on IL-3 contacting both IL-3Rα and β_c. To study whether IL-3 binding to both chains of the IL-3R was a prerequisite for IL-3R complex formation, we performed coimmunoprecipitation experiments after preincubation of the cells with MAb 7G3, which blocks IL-3 binding to IL-3Rα (46), and in the presence of the IL-3 mutant E22R, which has defective β_c interaction (2). We found that preincubation of cells with the blocking MAb (7G3) greatly reduced the abilities of the α chain to coimmunoprecipitate β_c (Fig. 4A) and of β_c to coimmunoprecipitate with IL-3Rα in the presence of IL-3 (Fig. 4B). Experiments comparing the effects of the IL-3 mutant E22R with wild-type IL-3 were performed over a range of ligand concentrations. We found that wild-type IL-3 induced the coimmunoprecipitation of IL-3Rα and β_c at a concentration of 0.1 nM and maximally at 1.0 nM (Fig. 5A and B). In contrast, the IL-3 mutant E22R did not cause coimmunoprecipitation of IL-3Rα and β_c at concentrations up to 100 nM (Fig. 5C and D). At higher concentrations (4 μM) of E22R, some coimmunoprecipitation of IL-3Rα and β_c was observed (data not shown). Again, a protein with an MW of approximately 80,000 coimmunoprecipitated with β_c in the absence of wild-type IL-3 (Fig. 5B) or in the presence of E22R (Fig. 5D) corresponding to GM-CSFRα. These experiments establish that IL-3 needs to contact both receptor chains in order to trigger receptor α and β_c association.

IL-3 induces disulfide- and nondisulfide-linked receptor dimers. To examine the possibility that IL-3 induces the covalent association of IL-3Rα and β_c, we next analyzed the immunoprecipitated proteins under nonreducing conditions. We found that in the absence of IL-3, the anti-IL-3Rα MAb immunoprecipitated only IL-3Rα (Fig. 6A). The anti-β_c MAb, on the other hand, immunoprecipitated mainly monomeric β_c but also consistently immunoprecipitated a faint band with an apparent MW of 245,000 (Fig. 6B). However, incubation with IL-3 not only led to the coimmunoprecipitation of IL-3Rα and β_c as shown above but also induced the appearance of two high-MW bands with apparent MW of approximately 215,000 and 245,000 (Fig. 6). This was observed whether immunoprecipitations were performed with anti-IL-3Rα MAb (Fig. 6A) or anti-β_c MAb (Fig. 6B). The high-MW complexes appeared to be disulfide linked, since treatment with the thiol-specific al-

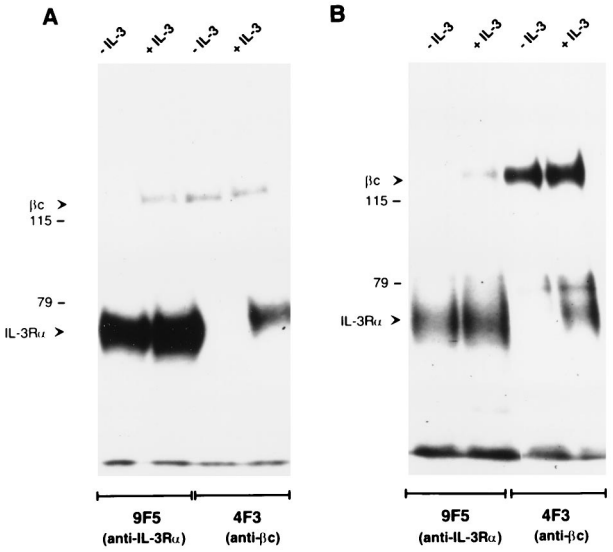

FIG. 2. IL-3 induces IL-3R complex formation in the human UT7 cell line and COS cells transfected with IL-3Rα and β_c. ^{125}I-surface-labelled cells UT7 cells (A) and COS cells transfected with IL-3Rα and β_c (B) were incubated with 50 nM IL-3 (+IL-3) for 15 min at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti-IL-3Rα) or MAb 4F3 (anti-β_c), separated on SDS–7.5% polyacrylamide gels under reducing conditions, and visualized by PhosphorImaging. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

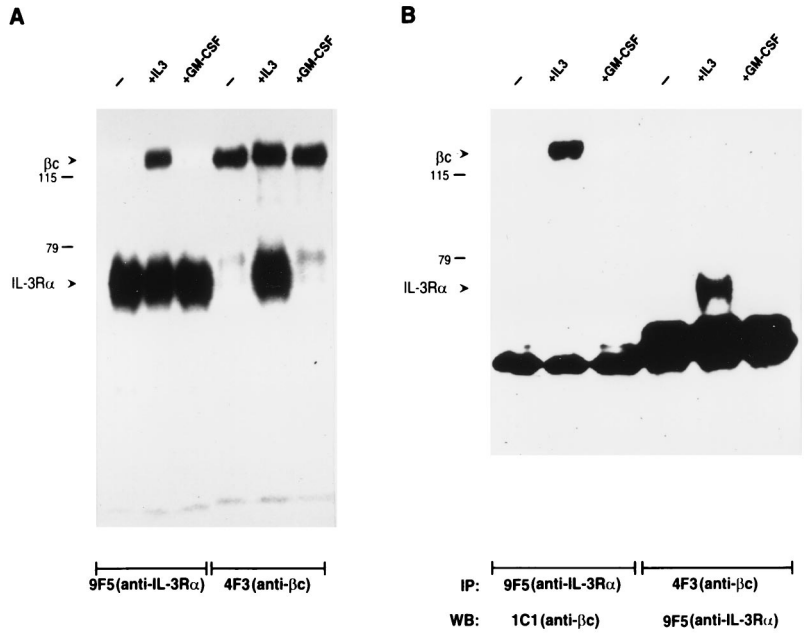

FIG. 3. Identification of coimmunoprecipitated bands as IL-3Rα and β_c by Western blotting. (A) Immunoprecipitations using MAb 9F5 (anti-IL-3Rα) or 4F3 (anti-β_c) and SDS-PAGE as described in the legend to Fig. 1 from cells not treated (−) or treated (+) with IL-3 (50 nM) or GM-CSF (50 nM) for 45 min at 4°C and visualized by PhosphorImaging. (B) Western blot of panel A using MAb 1C1 (anti-β_c) or MAb 9F5 (anti-IL-3Rα). The major band shows the immunoreactivity of the immunoprecipitating MAb. The positions and MW (in thousands) of marker proteins are shown to the left of the gels. IP, immunoprecipitation; WB, Western blotting.

kylating agent iodoacetamide prior to the addition of IL-3 inhibited their appearance (Fig. 6A and B, lanes IAM+IL-3). Despite the marked loss of the high-MW complexes, iodoacetamide pretreatment did not prevent IL-3Rα and β_c coimmunoprecipitation (Fig. 6A and B, lanes IAM+IL-3). Thus, IL-3 induces IL-3Rα and β_c association by covalent and noncovalent means. For controls, we used iodoacetamide after IL-3 treatment in which case little or no reduction in the formation of the high-MW complexes was observed (Fig. 6A and B, lanes IL-3+IAM). Furthermore, the addition of IL-3 to the lysis buffer did not induce IL-3Rα and β_c association (Fig. 6A and B, lanes lysate+IL-3), indicating that α and β_c association was not induced in solution. These controls demonstrate that the appearance of the high-MW complexes requires free sulfhydryl groups and IL-3 acting on the intact cells.

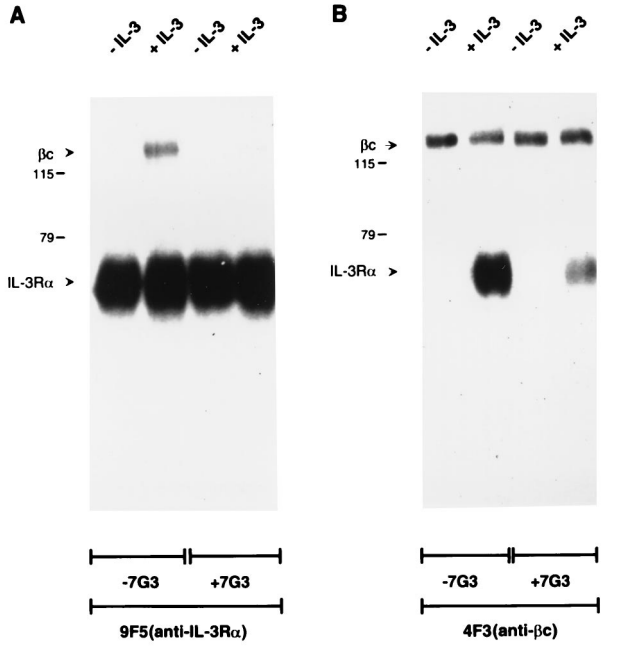

FIG. 4. The anti-IL-3Rα blocking MAb 7G3 inhibits IL-3Rα and β_c dimerization. ^{125}I-surface-labelled cells were preincubated with (+) 1 μg of MAb 7G3 for 30 min at 4°C before treatment with (+) or without (−) IL-3 (50 nM) for 45 min at 4°C. The cells were then lysed, and the lysates were subjected to immunoprecipitation with MAb 9F5 (anti-IL-3Rα) (A) or MAb 4F3 (anti-β_c) (B) as described in the legend to Fig. 1. The positions and MW (in thousands) are shown to the left of the gels.

We next examined whether IL-3 itself was present in the immunoprecipitated complexes. We did not perform Western blotting with anti-IL-3 antibodies, since it was possible that IL-3 epitopes could be masked by bound receptor. Instead, we stimulated the cells with radiolabelled IL-3 in the absence or presence of a 100-fold excess of unlabelled IL-3. We found that ^{125}I-labelled IL-3 could be immunoprecipitated with MAb 9F5 (anti-IL-3Rα) or MAb 4F3 (anti-β_c) and this immunoprecipitation was inhibited by excess unlabelled IL-3 (Fig. 6C). However, IL-3 was not covalently bound to IL-3Rα or β_c, as demonstrated by its migration to the 15,000-MW region (Fig. 6C).

Significantly, prevention of disulfide-linked dimer formation by iodoacetamide did not affect high-affinity binding of IL-3. Scatchard analysis of cells treated with iodoacetamide showed that high-affinity binding was essentially the same as that seen with untreated cells (Fig. 6D).

To identify the components of the disulfide-linked high-MW bands, we next performed 2D SDS-PAGE and Western blot analysis. 2D gel electrophoresis of immunoprecipitates from ^{125}I-labelled, IL-3-treated cells with MAb 9F5 (anti-IL-3Rα) and MAb 4F3 (anti-β_c), in which the nonreduced samples were separated in the first dimension and then reduced before separation in the second dimension, were performed. The two major proteins seen on the diagonal had MW of 120,000 and 70,000, corresponding to the MW of β_c and IL-3Rα, respec-

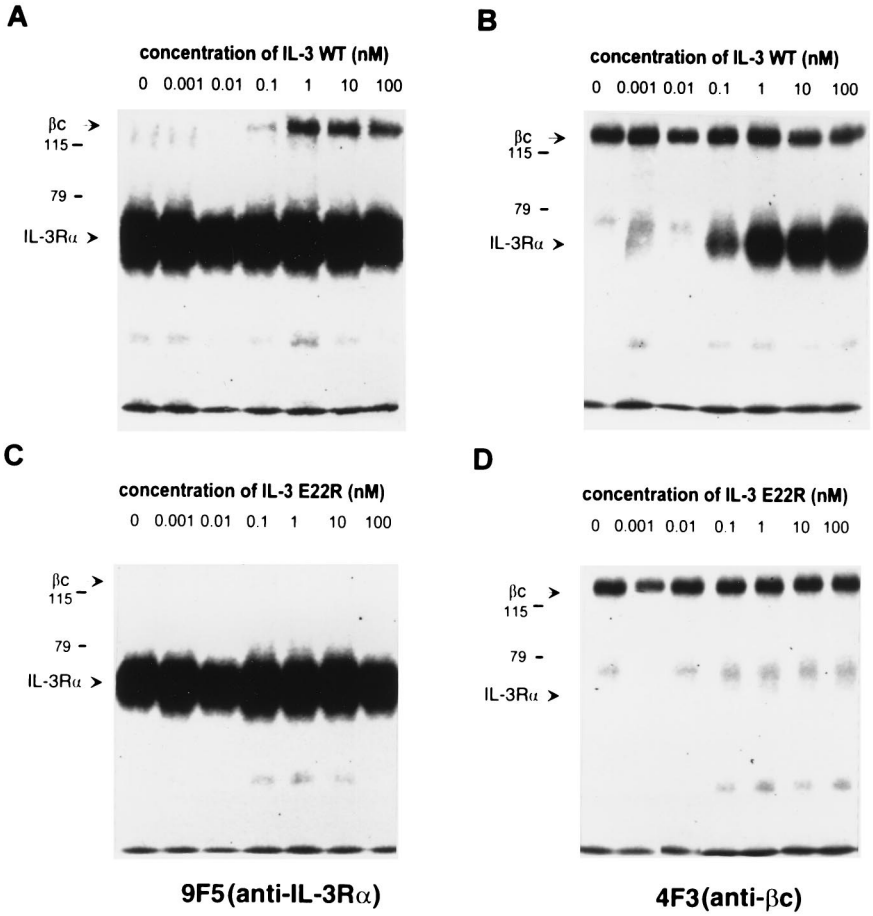

FIG. 5. The IL-3 analog E22R exhibits deficient induction of IL-3Rα and β_c dimerization. ^{125}I-surface-labelled cells were treated with different concentrations of wild-type (WT) IL-3 or with the IL-3 mutant E22R. The cells were then lysed, and the lysates were subjected to immunoprecipitation with MAb 9F5 (anti-IL-3Rα) (A and C) or MAb 4F3 (anti-β_c) (B and D) as described in the legend to Fig. 1. The positions and MW (in thousands) of marker proteins are shown to the left of the gels.

tively (Fig. 7). The 215,000-MW band in the nonreduced first dimension was resolved into two spots with MW of 120,000 and 70,000 in the reduced dimension, consistent with the MW of β_c and IL-3Rα, respectively. A broad, higher-MW complex in the nonreduced dimension was also resolved into 120,000- and 70,000-MW components, although this was less distinct than for the 215,000-MW complex. These experiments suggested that the two high-MW bands seen under nonreducing conditions contain IL-3Rα and β_c. To examine this identity further, we performed Western blotting of IL-3-stimulated cells immunoprecipitated with anti-IL-3Rα and anti-β_c MAb. In the absence of IL-3, no high-MW bands were immunoprecipitated with anti-IL-3Rα MAb (Fig. 8A), while a 245,000-MW band could be immunoprecipitated with anti-β_c MAb (Fig. 8A) and also contained IL-3Rα (Fig. 8B), as judged by Western blotting. After stimulation with IL-3, however, both MAb 9F5 (anti-IL-3Rα) and MAb 1C1 (anti-β_c) reacted with the 215,000- and 245,000-MW bands, confirming that IL-3Rα and β_c were both present in these disulfide-linked complexes (Fig. 8B and C).

IL-3-induced disulfide-linked dimers are required for receptor activation. In order to determine the functional consequences of IL-3R dimerization, we examined the immunoprecipitated complexes for the presence of phosphorylated tyrosine. We performed immunoprecipitations on ^{125}I-surface-labelled cells with anti-IL-3Rα and anti-β_c antibodies and probed the immunoprecipitates, separated under nonreducing or reducing conditions, with antiphosphotyrosine antibodies by Western blotting. The results showed that the antiphosphotyrosine MAb 3-365-10 reacted strongly with 215,000- and 245,000-MW bands (>94% of phosphotyrosine), but little label (<6% of phosphotyrosine) was observed in the 120,000-MW region. This antiphosphotyrosine staining was observed with immunoprecipitates by using either MAb 9F5 (anti-IL-3Rα) or 4F3 (anti-β_c) and disappeared when αβ covalent dimer formation was prevented by iodoacetamide (Fig. 9A). These results show that phosphorylation in response to IL-3 occurs primarily on the covalent αβ heterodimer, suggesting that αβ heterodimer formation is required for cellular activation. To ascertain whether both IL-3Rα and β_c were being phosphorylated, the immunoprecipitates with anti-IL-3Rα and anti-β_c MAb were separated under reducing conditions before analysis by Western blotting with the antiphosphotyrosine antibody 3-365-10. Under these conditions, only a phosphorylated band with an MW of about 120,000 was observed, consistent with β_c but not IL-3Rα being the phosphorylated protein present in the receptor dimer (Fig. 9A).

We further confirmed the predominant phosphorylation of the covalent αβ heterodimer by using the human UT7 cell line (Fig. 9B). The onset of phosphorylation in the covalent αβ

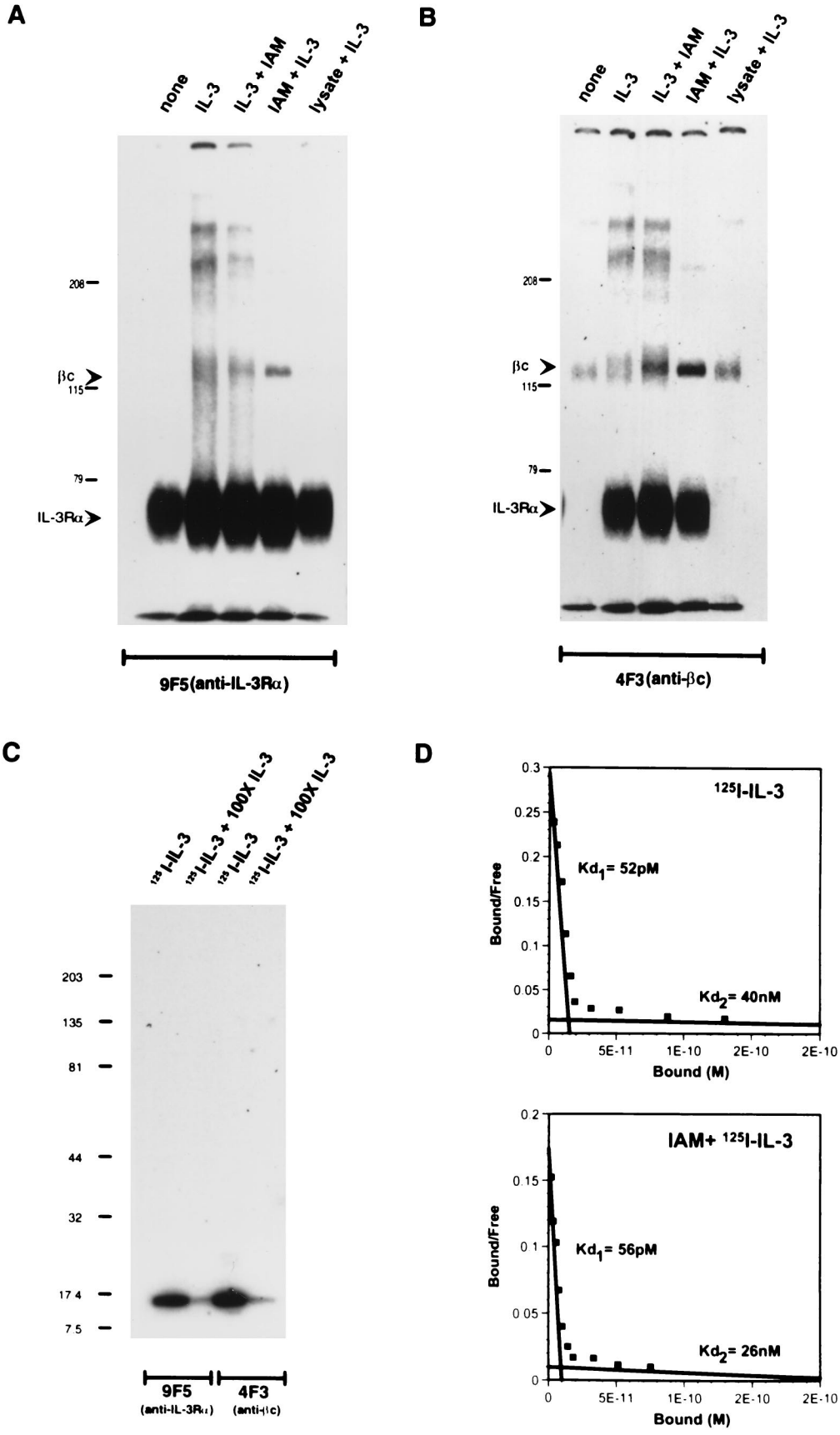
A
none
IL-3
IL-3 + IAM
IAM + IL-3
lysate + IL-3
208
βc
115
79
IL-3Rα
9F5(anti-IL-3Rα)
B
none
IL-3
IL-3 + IAM
IAM + IL-3
lysate + IL-3
208
βc
115
79
IL-3Rα
4F3(anti-βc)
C
125I-IL-3
125I-IL-3 + 100X IL-3
125I-IL-3
125I-IL-3 + 100X IL-3
203
135
81
44
32
17.4
7.5
9F5
(anti-IL-3Rα)
4F3
(anti-βc)
D
125I-IL-3
Kd1= 52pM
Kd2= 40nM
Bound/Free
Bound (M)
IAM+ 125I-IL-3
Kd1= 56pM
Kd2= 26nM
Bound/Free
Bound (M)

FIG. 6. IL-3 induces the formation of disulfide-linked receptor complexes. (A and B) From left to right, the lanes contain ^{125}I-surface-labelled cells incubated with either medium for 30 min at 37°C, IL-3 (50 nM) for 30 min at 37°C, IL-3 (50 nM) for 30 min at 37°C and then with iodoacetamide (IAM) (10 mM) for 5 min at 21°C, iodoacetamide (10 mM) for 5 min at 21°C and then with IL-3 (50 nM) for 30 min at 37°C, or medium only and IL-3 (50 nM) added to the cell lysate for 30 min at 4°C. Immunoprecipitations were carried out with MAb 9F5 (anti-IL-3Rα) (A) and with MAb 4F3 (anti-β_c) (B). (C) Unlabelled cells were incubated with ^{125}I-labelled IL-3 (1 nM) in the absence or presence of 100-fold-excess (100×) unlabelled IL-3 before immunoprecipitation with MAb 9F5 (anti-IL-3Rα) and MAb 4F3 (anti-β_c). The immunoprecipitated proteins were separated under nonreducing conditions on an SDS–6% polyacrylamide gel. (D) Scatchard transformation of ^{125}I-labelled IL-3 binding curves in untreated cells (top) or cells treated with iodoacetamide (10 mM) for 20 min at 4°C (bottom).

heterodimer was rapid and detectable at 1 min (Fig. 9B). The proportions of tyrosine phosphorylation in these heterodimers at 1, 5, and 15 min were 91, 87, and 82%, respectively. As with CML cells, the formation of the covalent αβ heterodimer was prevented and tyrosine phosphorylation was abolished by pretreatment of UT7 cells with iodoacetamide (data not shown). To control for any possible toxic effects of iodoacetamide, UT7 cells were pretreated with this agent and then stimulated with phorbol myristate acetate and calcium ionophore. Under the conditions used, no inhibition of basal or induced tyrosine phosphorylation was observed (Fig. 9C), indicating that iodoacetamide was not toxic to the cells.

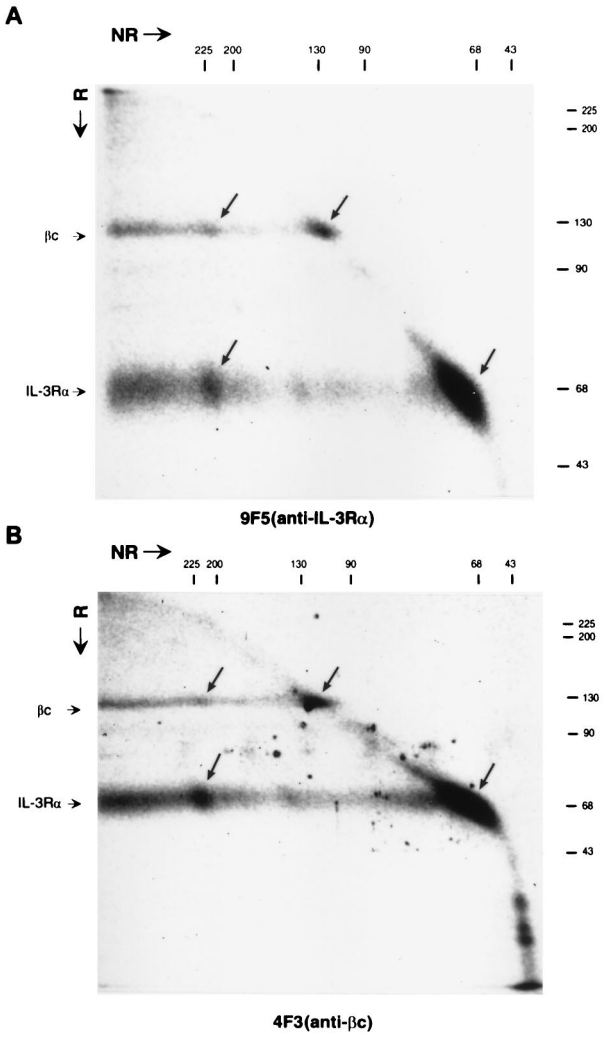

FIG. 7. 2D gel electrophoresis of ^{125}I-surface-labelled cells incubated with 50 nM IL-3 for 30 min at 4°C and immunoprecipitated with anti-IL-3Rα MAb (9F5) (A) or anti-β_c MAb (4F3) (B). The immunoprecipitates were separated under nonreducing (NR) conditions in the first dimension and under reducing (R) conditions in the second dimension and visualized by PhosphorImaging. The immunoprecipitates were mixed with unlabelled platelet extract and stained with Coomassie blue (not shown) to obtain more accurate MW estimates. The positions and MW (in thousands) of proteins are shown at the sides of the gels.

DISCUSSION

We show here that human IL-3 binding to its receptor triggers the heterodimerization of its specific receptor binding subunit, IL-3Rα, with β_c and that receptor heterodimerization is dependent on IL-3 contacting both receptor subunits. Both disulfide-linked and non-disulfide-linked heterodimers were observed and, although IL-3 is required for their formation, IL-3 is not covalently attached to the dimers. Importantly, the disulfide-linked heterodimer and not the noncovalently linked heterodimer is shown to be required for receptor activation but not high-affinity binding. These results are different from those in the IL-6, EPO, and G-CSF receptor systems where receptor activation involves homodimerization of a single signalling subunit and may apply also to the related GM-CSF and IL-5 receptors.

Previous experiments have shown that IL-3R activation leads to stimulation of JAK-2 (43) and Lyn (48) kinases, as well as the Ras–mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C pathways (41). The molecular basis of receptor activation has not been revealed, although by analogy with other receptor systems, it is believed to involve receptor dimerization. Receptor dimerization has been demonstrated in tyrosine kinase receptors (14, 49), as well as the G-CSFR (16), EPO receptor (28), IL-6R (32), and CNTFR (7). Dimerization of the IL-3R, GM-CSFR, or IL-5R has been proposed in ligand-induced and ligand-independent receptor activation (6, 17), but no evidence has been forthcoming. The results presented here demonstrate both covalent and noncovalent dimerization of the receptor, that it involves the ligand-binding subunit dimerizing with β_c, and that covalent linkage of dimers is required for signalling. These results emphasize the importance of the α chain not only as a ligand-binding subunit but also as a subunit required for signalling in primary cells. This is consistent with experiments where deletion of the cytoplasmic domain of α chains abolishes signalling (40, 54). Other experiments have shown, however, that the cytoplasmic domain of β_c can substitute for the intracytoplasmic domain of the α chain (9, 33, 47). The exact significance of these findings is not clear but these findings may reflect the conserved nature of the proline-rich sequence present in the membrane-proximal domain of both the α chain and β_c or the fact that at least two molecules of β_c are required for signalling, with the role of the α chain being to recruit or facilitate the association of two molecules of β_c. Our experiments demonstrating the presence of both IL-3Rα and β_c in the 215,000- and 245,000-MW complexes suggest that a heterodimer containing one α chain and one β_c may be sufficient for signalling, although we cannot rule out the existence of higher-MW oligomeric complexes. Definition of the stoichiometry of the active receptor complex would require direct measurements using purified receptor components as shown with the IL-6R (35, 51).

The presence of IL-3Rα in the disulfide-linked IL-3R dimers

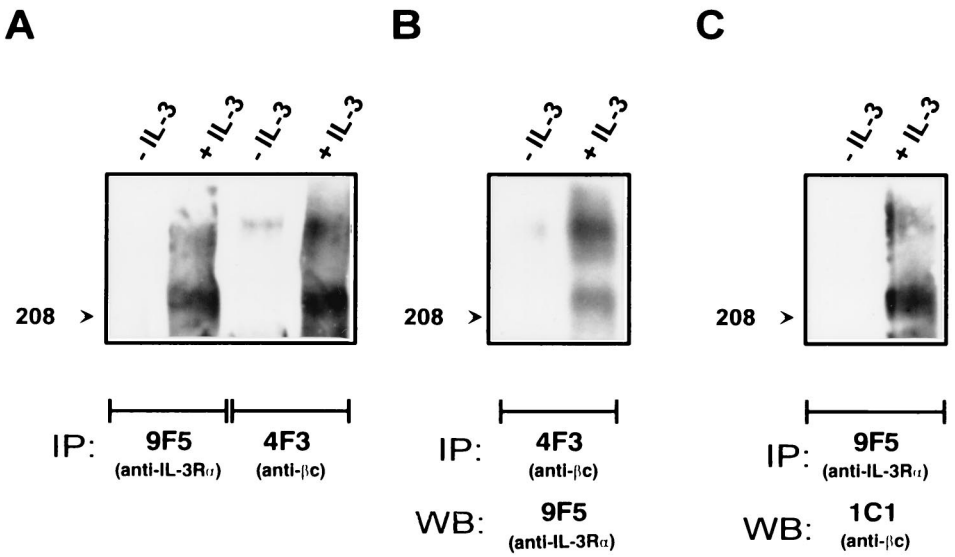

FIG. 8. IL-3-induced disulfide-linked complexes contain IL-3Rα and β_c. ^{125}I-surface-labelled cells were treated and immunoprecipitated as described in the legend to Fig. 6. (A) Immunoprecipitation (IP) with MAb-9F5 (anti-IL-3Rα) and MAb 4F3 (anti-β_c). (B) Immunoprecipitates with MAb 4F3 (anti-β_c) were analyzed by Western blotting (WB) with MAb 9F5 (anti-IL-3Rα). (C) Immunoprecipitates with MAb 9F5 (anti-IL-3Rα) were analyzed by Western blotting with MAb 1C1 (anti-β_c). Proteins were separated as described in the legend to Fig. 6. The position and MW (in thousands) of marker protein are shown to the left of the gels.

contrasts with the disulfide-linked dimerization of the IL-6 and CNTF receptors which involve only the signal transducer subunits. Thus, in the case of the IL-6R, disulfide-linked dimerization involves only gp130 (7), while in the case of the CNTFR, gp130 and LIFR form disulfide-linked dimers (7), events which appear to initiate signal transduction. These findings suggest a fundamental difference in the functional contribution of IL-3Rα compared with IL-6Rα and CNTFRα in receptor activation and suggest that IL-3Rα is not only involved in the initial binding of ligand but also participates in signalling. These results are consistent with differences in the requirement for the cytoplasmic domain of the α chains for signalling. Thus, while the cytoplasmic domain of the α chains of the IL-3, GM-CSF, and IL-5 subfamily of receptors are essential for signal transduction (37, 40), the cytoplasmic portion of IL-6Rα is not (57). On the other hand, IL-3Rα behaves like the LIFR, which participates both in ligand binding and signalling.

Human IL-3R heterodimerization upon addition of IL-3 was found to be very rapid (it is complete within 1 min) and dose dependent. An anti-IL-3Rα monoclonal antibody which inhibits IL-3 binding to this chain (46) prevented IL-3-induced receptor heterodimerization. Similarly, the IL-3 mutant E22R which is selectively deficient at interacting with β_c (2) failed to induce receptor dimerization in a concentration range in which wild-type IL-3 did so. This indicates that IL-3 needs to contact both receptor chains to stabilize the complex and induce receptor dimerization.

Under reducing conditions of SDS-PAGE, the IL-3-induced dimers were resolved into monomeric IL-3Rα and β_c. Under nonreducing conditions, however, two high-MW complexes with MW of 215,000 and 245,000 were also seen in immunoprecipitations performed with both anti-IL-3Rα and anti-β_c MAb. These were disulfide-linked dimers, as judged by their disappearance in the presence of the thiol-specific alkylating agent iodoacetamide added before but not after incubation with IL-3. In the absence of IL-3, a faint band with an apparent MW of about 245,000 was consistently seen, but only in immunoprecipitations performed with MAb anti-β_c (Fig. 6 and 8). Western blotting showed that this band contains β_c (data not shown) and IL-3Rα (Fig. 8B), suggesting that a small proportion of receptors may exist as preformed, though inactive (see below) dimers. IL-3 was present in the IL-3-induced receptor dimers, although it was not disulfide linked to either receptor chain (Fig. 6).

The sizes of the two high-MW complexes suggested that they might represent IL-3Rα and β_c heterodimers or β_c homodimers. By performing 2D SDS-PAGE (Fig. 7) and Western blotting (Fig. 8) experiments, we could show that both IL-3Rα and β_c were present in the 215,000- and 245,000-MW bands. In addition, the 2D SDS-PAGE suggested that higher-MW complexes were also formed. The presence of both IL-3Rα and β_c in these complexes may account for the 215,000-MW band, but the 245,000-MW band may contain another protein in addition to IL-3Rα and β_c. This accessory protein is not IL-3, as IL-3 is not disulfide linked to either receptor chain (see above). It is possible that a third, poorly iodinated surface protein or a small cytoplasmic protein becomes covalently linked to the IL-3R upon activation, but its presence and identity remain to be determined.

The presence of IL-3-induced disulfide-linked and non-disulfide-linked heterodimers suggests two types of IL-3Rα and β_c interaction, a noncovalent one and one that is mediated by Cys-Cys bridging of the receptors. The reason for the presence of both types of complexes or the sequence of their appearance is not clear. It is possible that initially IL-3 binds to IL-3Rα and the IL-3–IL-3Rα complex binds β_c, forming a high-affinity complex. IL-3 high-affinity binding then triggers a noncovalent association, with both events being mediated by cytokine receptor module 2 (CRM2) in β_c (Fig. 10). The main function of these events may be then to bring IL-3Rα and β_c into close proximity, thus facilitating a proportion of the dimers undergoing disulfide linkage through CRM1 of β_c (Fig. 10). These disulfide-linked dimers are therefore not required for high-affinity binding but instead are then involved in recruiting tyrosine kinases which phosphorylate dimerized β_c and intracellular proteins. Alternatively, the association of IL-3Rα and β_c may be primarily covalent, but after SDS-PAGE, a propor-

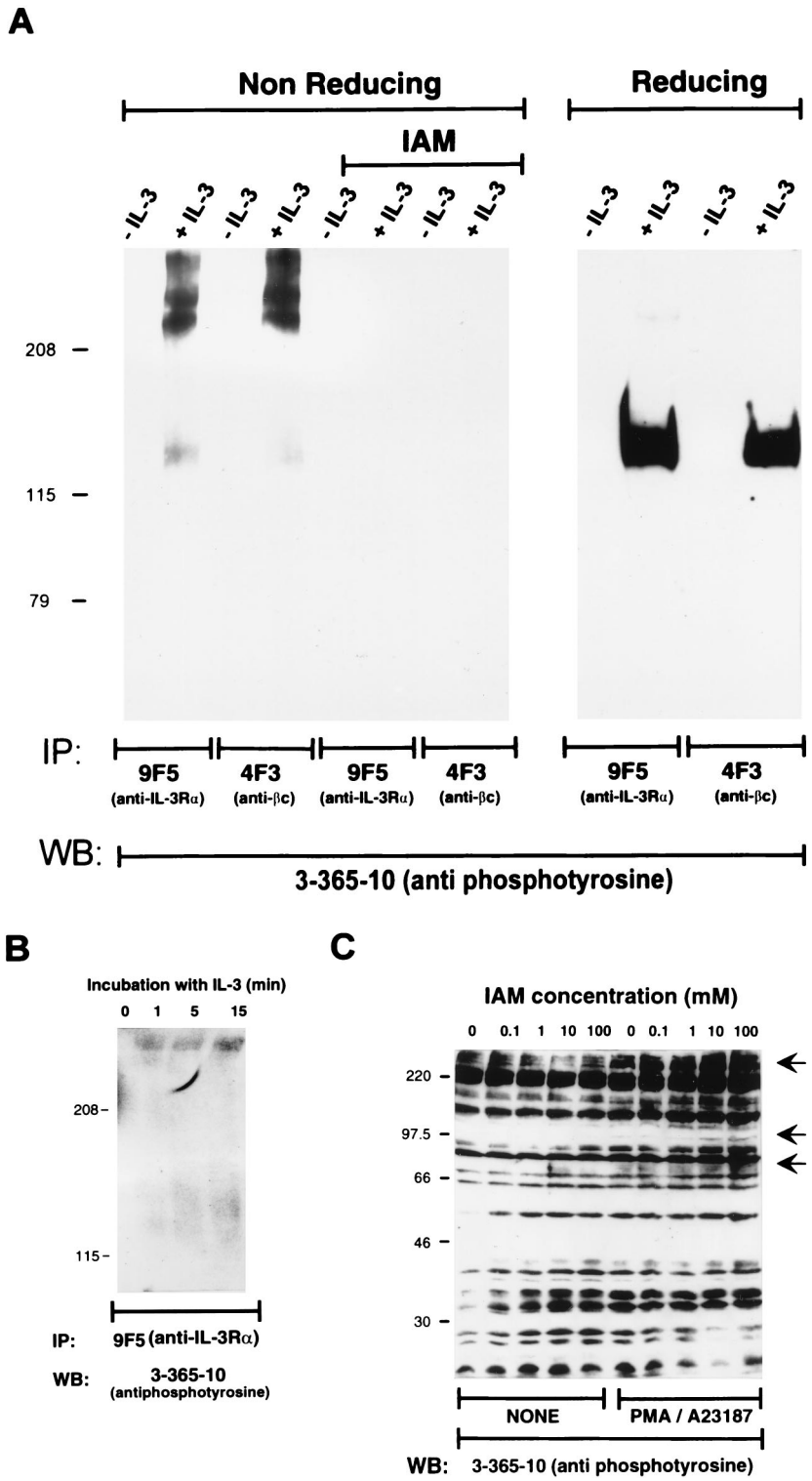

FIG. 9. Tyrosine phosphorylation of disulfide-linked receptor dimers. (A) Unlabelled CML cells were either not treated (−IL-3) or incubated with IL-3 (50 nM) (+IL-3) for 30 min at 4°C with or without the prior addition of iodoacetamide (IAM) and immunoprecipitated with MAb 9F5 (anti-IL-3Rα) or MAb 4F3 (anti-β_c). The immunoprecipitates were separated under nonreducing or reducing conditions on SDS–6% polyacrylamide gels and probed with the antiphosphotyrosine antibody 3-365-10. (B) Unlabelled UT7 cells were incubated with 50 nM IL-3 for different times at 4°C. After cell lysis, proteins were immunoprecipitated with MAb 9F5 (anti-IL-3Rα) and separated under nonreducing conditions on an SDS–6% polyacrylamide gel and probed with the antiphosphotyrosine antibody 3-365-10. (C) Human UT7 cells were not treated or treated with various concentrations of iodoacetamide for 20 min at 4°C, and then either not stimulated or stimulated with phorbol myristate acetate (50 ng/ml) and calcium ionophore A23187 (2 μM) (PMA/A23187) for 60 min at 4°C. After cell lysis, proteins were separated under reducing conditions on SDS–12.5% polyacrylamide gels and probed with the antiphosphotyrosine antibody 3-365-10. The arrows indicate the positions of the newly induced proteins. MW (in thousands) of marker proteins are shown to the left of the gels. IP, immunoprecipitation; WB, Western blotting.

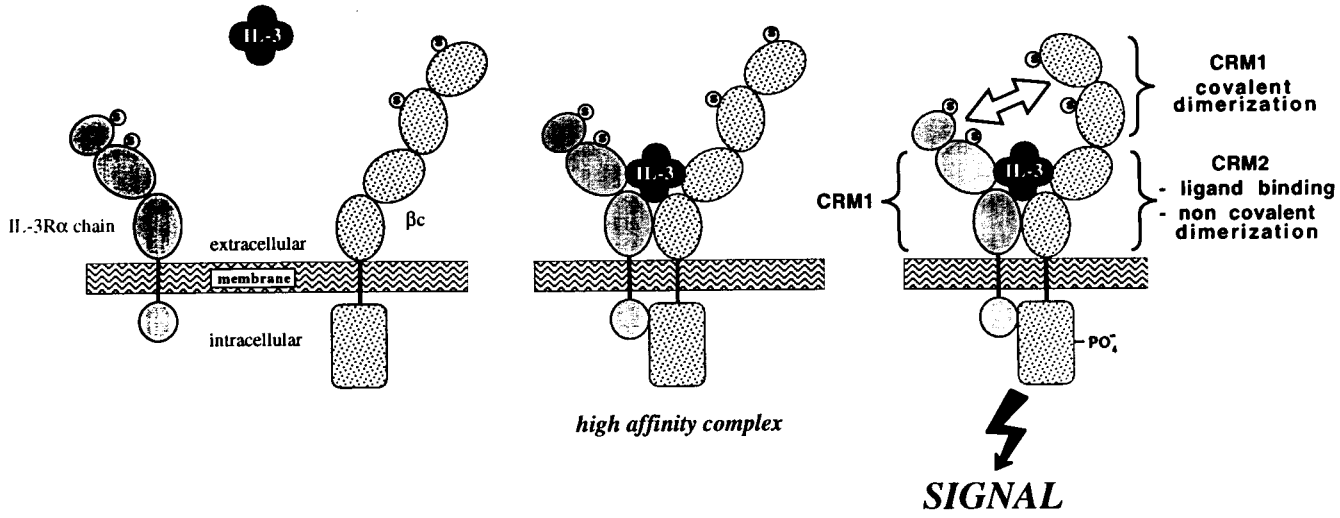

FIG. 10. Model of human IL-3R activation. In the absence of IL-3, IL-3Rα and β_c are not associated on the cell surface. The presence of IL-3 triggers IL-3 binding to IL-3Rα initially and then to β_c. IL-3 binding to β_c occurs through the cytokine receptor module CRM2 (55, 56) and triggers dimerization. Two complementary mechanisms of receptor heterodimerization are proposed: a noncovalent one probably involving the A-B loop in membrane-proximal domains of the CRM2 of each chain analogous to the growth hormone receptor (8) and a covalent association probably involving unpaired Cys residues in the N-terminal region and domain 1 of IL-3Rα interacting with hitherto unpaired Cys residues in the CRM1 of β_c (see Discussion). IL-3 is associated with the receptor dimer but is not covalently attached to it. Disulfide-linked receptor heterodimerization leads to phosphorylation of the dimerized β_c, but not to phosphorylation of noncovalently associated monomeric β_c or IL-3Rα, an event that leads to cellular activation.

tion of this complex is disrupted by intramolecular disulfide bond rearrangement.

We found that by preventing disulfide-linked dimer formation, it was possible to dissociate high-affinity binding from receptor activation. Thus, high-affinity binding could take place in the absence of receptor activation (Fig. 6 and 9). It may therefore be possible to construct IL-3 antagonists that retain full high-affinity binding but which are deficient at inducing disulfide-linked dimers. On the other hand, if high-affinity binding inevitably leads to disulfide-linked dimer formation and cellular activation, antagonism of IL-3 may rely largely on compounds that prevent disulfide-linked dimerization.

Two lines of evidence showed that disulfide-linked receptor dimerization is involved in receptor activation. First, the vast majority of the tyrosine-phosphorylated bands immunoprecipitated by anti-IL-3Rα or anti-β_c MAb corresponded to the disulfide-linked heterodimers (Fig. 9). This is even more remarkable when it is considered that while <17% of the IL-3-dependent IL-3Rα and β_c heterodimers are disulfide linked, >94% of the tyrosine phosphorylation is associated with these dimers. Second, blocking of the thiol groups with iodoacetamide prevented receptor phosphorylation under conditions that did not affect cell viability and total cellular phosphorylation (Fig. 9C) or high-affinity binding (Fig. 6D), although we cannot rule out inactivation of JAK-2. This is analogous to the IL-6 receptor, where the presence of gp130 homodimers is accompanied by tyrosine phosphorylation of these proteins (32). Therefore, the disulfide-linked heterodimerization of IL-3Rα and β_c appears to be essential for tyrosine phosphorylation, which is known to involve the Janus family of tyrosine kinases (43). Since both IL-3Rα and β_c contain intracellular tyrosine residues, we examined whether both receptor chains were being phosphorylated by reduction of the disulfide-linked dimers with dithiothreitol and probing with antiphosphotyrosine antibodies. This revealed that β_c but not IL-3Rα was the phosphorylated protein in the heterodimers (Fig. 9). This is consistent with preassociated JAK-2 causing phosphorylation of β_c (38, 43). It should be noted that although disulfide-linked dimerization appears essential for receptor phosphorylation, it is possible that other functions are not affected, and it would be of interest to examine functions, such as proliferation, under conditions that prevent disulfide-linked dimer formation. The identification of the cysteines involved in disulfide formation and their mutation may reveal IL-3 activation pathways that occur independently of this process.

The IL-3-induced appearance of disulfide-linked IL-3Rα and β_c dimers suggests the presence of free Cys in these molecules. Examination of the sequences of IL-3Rα and β_c and modelling of the cytokine receptor modules (11) suggest that IL-3Rα has two potentially unpaired Cys residues, one in the N-terminal domain at position 52, 68, or 76 and one at position 195 in domain 1 of the CRM (Fig. 10). Similarly, β_c has two potentially free Cys residues, one at position 91, 96, or 100 in domain 1 and one at position 234 in domain 2 of CRM1 (Fig. 10). It is interesting to note that, in contrast to the α chains, β_c has two CRMs. While CRM2 has been implicated in binding IL-3, GM-CSF, and IL-5 (23, 56), the function of CRM1 is unknown. We hypothesize that this is important for disulfide linkage and suggest that, analogous to the cytoplasmic domain of β_c (42), the extracellular region can be viewed as having two functional domains, with CRM2 involved in ligand recognition and nondisulfide-linked heterodimerization (Fig. 10) and CRM1 involved in the final activation step which includes αβ disulfide formation.

It is also worth noting that the N-terminal regions of IL-3Rα, GM-CSFRα, and IL-5Rα are significantly conserved between each other and are present only in this family of receptors (11). Although the number of Cys residues in this region varies in IL-3Rα (3 Cys), GM-CSFRα (5 Cys), and IL-5Rα (1 Cys), all three α chains exhibit one unpaired Cys. This raises the possibility that these unpaired Cys residues are involved in disulfide-linked dimerization and suggests a functional reason for the conservation of this N-terminal region in the IL-3Rα, GM-CSFRα, and IL-5Rα family.

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